Bioorganic & Medicinal Chemistry 20 (2012) 1584–1593

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Coumarin polysulfides inhibit cell growth and induce apoptosis in HCT116 colon cancer cells

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ARTICLE INFO

Article history:
Received 15 September 2011
Revised 14 December 2011
Accepted 17 December 2011
Available online 30 December 2011

Keywords:
Coumarin polysulfides
Apoptosis
Cell cycle arrest
p53
Phosphatase

ABSTRACT

Coumarins and coumarin derivatives as well as diallyl polysulfides are well known as anticancer drugs. In order to find new drugs with anticancer activities, we combined coumarins with polysulfides in the form of di-coumarin polysulfides. These novel compounds were tested in the HCT116 colorectal cancer cell line. It turned out that they reduced cell viability of cancer cells in a time and concentration dependent manner. Cells tested with these coumarin polysulfides accumulate in the G_2/M phase of the cell cycle and finally they go into apoptosis. A decrease in bcl-2 level, and increase in the level of bax, cytochrome *c* release into the cytosol, cleavage of caspase 3/7and PARP suggested that coumarin polysulfides induced the intrinsic pathway of apoptosis. Comparison of these new coumarin compounds with the well known diallyl polysulfides revealed that the coumarin disulfides were more active than the corresponding diallyl disulfides. The activities of the coumarin tetrasulfides and the corresponding diallyl tetrasulfides are similar. The novel coumarin compounds regulated the phosphatase activity of the cell cycle regulating cdc25 family members, indicating that these phosphatases are implicated in the induction of cell cycle arrest and possibly in apoptosis induction as well. In addition, coumarin polysulfides also down-regulated the level of cdc25C, which also contributed to the arrest in the G_2-phase of the cell cycle.

1. Introduction

Natural as well as synthetic coumarins have recently drawn much attention due to their broad pharmacological activities. Coumarins bear a 2*H*-1-benzopyran-2-one as part of their structure.[1] They show a wide range of biological activities, for example, as antioxidants,[2] anticoagulants,[3] antifungal agents,[4] monoamine oxidase B (MAO-B) selective inhibitors,[5,6] human acetylcholine esterase (hAChE) and beta-site amyloid precursor protein cleavage enzyme (BACE1) inhibitors,[7] NFB inhibitors,[8] Hsp90 inhibitors,[9] HIV-1 integrase inhibitors[10,11] and cdc25 phosphatases inhibitors.[12] Moreover, the coumarin scaffold has recently been reported to inhibit matrix metalloproteinase-7 expression,[13] 17β-hydroxysteroid-dehydrogenase type 1 (17-β-HSD1) activity,[14,15] as well as cannabinoid receptor antagonists.[16] The potential use of coumarin derivatives as anticancer agents has been well reviewed,[17] and moreover recently new studies have highlighted the interesting role of coumarin analogues in cancer therapy.[18–25] Therefore the coumarin scaffold remains of great interest for researchers.

Another class of compounds which are highly effective in affording protection against various cancers in animal models are the polysulfides. Organo sulfur compounds can afford protection against cancer induced by a variety of chemical carcinogens in animal models (for review see:[26]). Diallyl sulfides in particular are known to prevent cancer by multiple mechanisms including impairment of carcinogen activation, inhibition of post-translational modifications of proteins, induction of apoptosis, histone modifications, inhibition of angiogenesis and metastasis (for review see:[27]). It is also becoming clear that diallyl polysulfides in particular are promiscuous because they target multiple signal transduction pathways to trigger growth arrest and eventually apoptosis. The anti-proliferative effects of the polysulfides seem to be related to their capacity to cause oxidative damage by increasing the production of reactive oxygen species. Cells lacking antioxidant defences are quite susceptible to polysulfides, whereas, cells well equipped with antioxidant defences such as glutathione peroxidase, copper/zinc superoxide dismutase are resistant to at least diallyl disulfide.[28,29] The number of sulfur atoms in the polysulfides seems to be critical for the activity of the compounds. Replacement of the sulfur atoms in diallyl sulfides by carbon atoms results in inactive molecules.[30] The presence of the allyl group generally enhances protection over that provided by the propyl moiety. In order to find new drugs with anticancer

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doi:10.1016/j.bmc.2011.12.032

activities, we made use of both coumarin and polysulfide and designed the synthesis and biological evaluation of novel hybrid-type bis-coumarin polysulfides, as disulfur, trisulfur and tetrasulfur coumarins. These compounds were tested in human HCT116 colon cancer cells to assay the capability of inducing cell growth inhibition and apoptosis. We found that coumarin polysulfides effectively reduced cell viability in a concentration and time dependent manner. This reduction in cell viability is accompanied by cell cycle arrest in the G_2-phase of the cell cycle and induction of apoptosis. Down-regulation of the cdc25 phosphatase activity and the level of cdc25C protein seem to be responsible at least for the cell cycle arrest. A decrease in the level of bcl-2 and an increase in the level of bax, release of cytochrome c into the cytosol, caspase 3/7 activation and PARP cleavage strongly argue for the induction of the intrinsic pathway of apoptosis.

2. Materials and methods

2.1. Synthesis of coumarin sulfides

The solvents used for the synthesis of coumarin sulfides were purchased from Carlo Erba (Val de Reuil, France) and the reactives from Acros Organics (Illkirch, France). Melting points were determined with a Stuart SMP3 apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AC 250 MHz spectrometer in $CDCl_3$ or DMSO-d_6. Mass spectra were recorded with a MicroTof-Q 98. Chemical shifts are reported in δ (ppm) units relative to the internal reference tetramethylsilane (Me_4Si). All reactions were routinely checked by TLC. TLC was performed on aluminum-backed silica gel plates (Merck DC, Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light. Tetrahydrofuran was distilled from sodium and benzophenone (Scheme 1).

2.2. Synthesis of 4-mercaptomethylchromen-2-one (2)

An equimolar mixture of 4-chloromethylcoumarin **1** (4.45 mmol, 1 g) and thiourea (4.45 mmol, 0.339 g) was stirred in ethanol/diethylether (1:1) mixture at room temperature for 2 h and then refluxed on a steam-bath overnight. After the separated solid was filtered, washed with diethylether (3 × 20 ml), dissolved in 5% NaOH (15 ml), filtered and the filtrate was re-precipitated with 2 N HCl. The solid was washed with water (3 × 15 ml) and diethylether (3 × 15 ml) to provide pure **2** as a colorless solid that was recrystallized in ethanol; mp: 150–152; Yield: 75%; ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.78–1.84 (t, 1H, –*SH*), 3.70–3.74 (d, 2H, –C*H*$_2$SH), 3.85 (s, 3H, –OC*H*$_3$), 6.25 (s, 1H, –C*H*COO–), 6.77–6.84 (m, 2H, benzene protons), 7.47–7.51 (d, 1H, benzene proton) ppm; ^{13}C NMR (250 MHz, $CDCl_3$) δ 25.11, 55.81, 101.24, 111.27, 112.56, 125.22, 153.98, 155.86, 161.16, 162.84 ppm; HRMS (ESI) $[M+Na]^+$ $C_{10}H_8NaO_2S$ calcd 215.0245, found 215.0250.

2.3. Synthesis of 4,4′-disulfanediylbis(methylene)bis(2*H*-chromen-2-one) (SV25)

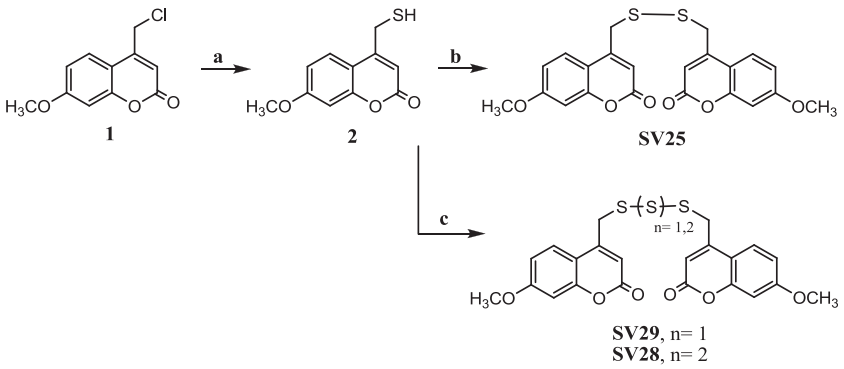
A suspension of 4-mercaptomethylcoumarin (0.90 mmol, 0.2 g) and manganese dioxide (4.50 mmol, 0.391 g) in tetrahydrofuran (10 ml) was refluxed for 1 h. The solid was then filtered and the filtrate was concentrated and recrystallized by EtOH to obtain pure SV25 as a pale yellow solid; mp: 140–142 °C; Yield: 72%; ^{1}H NMR (250 MHz, DMSO-d_6) δ 3.85 (s, 6H, –OC*H*$_3$), 4.06 (s, 4H, –C*H*$_2$S–), 6.09 (s, 2H, –C*H*COO–), 6.91–6.99 (m, 4H, benzene protons), 7.75–7.79 (d, 2H, benzene protons) ppm; ^{13}C NMR (250 MHz, $CDCl_3$) δ 39.07, 55.85, 101.53, 110.83, 112.20, 113.21, 125.52, 149.52, 155.79, 160.37, 163.01 ppm; HRMS (ESI) $[M+Na]^+$ $C_{22}H_{18}NaO_6S_2$ calcd 465.0545, found 465.0551.

2.4. General procedure for the synthesis of 4,4′-tri- and tetrasulfanediylbis(methylene)bis(7-methoxy-2*H*-chromen-2-one) (SV29 and SV28) Example: synthesis of 4,4′-trisulfanediylbis (methylene)bis(7-methoxy-2H-chromen-2-one) (SV29)

N,N-diisopropylethylamine (0.81 mmol, 0.14 ml), and sulfur dichloride (0.337 mmol, 0.021 ml) were added at 0 °C to a solution of 4-mercaptomethylchromen-2-one **2** (0.675 mmol, 0.150 g) in tetrahydrofuran (10 ml) and the resulting mixture was stirred at 0 °C for 1 h. The reaction was then quenched by water (50 ml) and the precipitate was filtered, washed with water (3 × 10 ml), diethylether (3 × 10 ml) and thereafter, recrystallized in EtOH/THF; mp: 153–155; Yield: 68%; ^{1}H NMR (250 MHz, DMSO-d_6) δ 3.85 (s, 6H, –OC*H*$_3$), 4.06 (s, 4H, –CH$_2$S–), 6.09 (s, 2H, –C*H*COO–), 6.91–6.99 (m, 4H, benzene protons), 7.75–7.79 (d, 2H, benzene protons) ppm; ^{13}C NMR (250 MHz, $CDCl_3$) δ 39.07, 55.85, 101.53, 110.83, 112.20, 113.21, 125.52, 149.52, 155.79, 160.37, 163.01 ppm; HRMS (ESI) $[M+Na]^+$ $C_{22}H_{18}NaO_6S_3$ calcd 497.0266, found 497.0270. For SV28: recrystallized by EtOH/THF; mp: 126–128; Yield: 73%; ^{1}H NMR (250 MHz, DMSO-d_6) δ 3.85 (s, 6H, –OC*H*$_3$), 4.06 (s, 4H, –CH$_2$S–), 6.09 (s, 2H, –C*H*COO–), 6.91–6.99 (m, 4H, benzene protons), 7.75–7.79 (d, 2H, benzene protons) ppm; ^{13}C NMR (250 MHz, $CDCl_3$) δ 39.07, 55.85, 101.53, 110.83, 112.20, 113.21, 125.52, 149.52, 155.79, 160.37, 163.01 ppm; HRMS (ESI) $[M+Na]^+$ $C_{22}H_{18}NaO_6S_4$ calcd 528.9884, found 528.9889.

2.5. Reagents and antibodies

Protease inhibitor cocktail Complete™ was purchased from Roche Diagnostics, Mannheim, Germany, *N*-acetyl cysteine (NAC)



Scheme 1. Reaction scheme for the synthesis of coumarin sulfides. Reagents and conditions: (a) (1) thiourea, EtOH/Et_2O, reflux, overnight; (2) 2 N NaOH; (3) 2 N HCl; (b) MnO_2, dry THF, 70 °C, 1 h; (c) Et_3N, sulfur dichloride or disulfur dichloride, 0 °C, 1 h.

and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies against p53, bax, bcl-2, cdc25C (H6), cytochrome *c* and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against caspase 3/7 was purchased from Promega (Mannheim, Germany). Anti-poly(-ADP-ribose) polymerase (PARP) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA) and a rabbit serum against nucleolin was prepared in our laboratory.[31]

2.6. Cell culture

p53-Positive HCT116 cells were maintained at 37 °C and 5% CO_2 in McCoy's 5A medium (PromoCell, Heidelberg, Germany) with 10% fetal calf serum (FCS). LNCaP cells were cultured to obtain positive control lysates for apoptosis induction. These cells were maintained at 37 °C and 5% CO_2 in RPMI 1640 medium supplemented with 10% FCS. Coumarin polysulfides and diallyl polysulfides were dissolved in DMSO to a 80 mM stock solution, which was freshly prepared before use. NAC was dissolved in distilled water to a 80 mM stock solution and applied to the cell culture medium 0.5 h (unless otherwise stated) before treatment. Doxorubicin was dissolved in distilled H_2O to a stock solution (10 mg/ml). Cells were treated with the coumarin polysulfides or doxorubicin at the indicated final concentrations for different times.

2.7. Evaluation of cell viability

In order to determine the effect of coumarin polysulfides on HCT116 colorectal cells, cells were seeded at 1×10^4 cells per well to a final volume of 500 μl in a 24-well plate and incubated overnight. Cells were then incubated in various concentrations of coumarin polysulfides for 8, 24 and 48 h. Viability of the cells was determined by a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay according to the manufacturer's instructions. One hour before the end of treatment, 50 μl MTT (5 mg/ml PBS) were added. The enzymatic reaction took place at 37 °C in a humified atmosphere. Following 1 h MTT treatment, medium was disposed off and cells solubilized by adding 500 μl solubilizing solution (0.05% (w/v) SDS in DMSO and 0.01% acetic acid) to each well and allowing the crystals to completely dissolve. The spectrophotometrical absorbance of the purple–blue formazan dye was determined in an ELISA reader at 595 nm.

2.8. Cell cycle analysis

HCT116 cells (5×10^4) were allowed to grow on a 10 cm petri dish overnight. The medium was changed and cells were treated with 0 (control), 25 or 50 μM SV29 and incubated for 24 h. Cells were collected and washed two times with cold PBS before been resuspended in PBS and fixed with 70% ethanol. The cells were further incubated with RNase and propidium iodide to label DNA as previously described.[30] Cells were then analyzed in a cytofluorimeter (Guava easyCyte HT system, Millipore) according to the manufacturer's instructions.

2.9. Treatment with antioxidant

To explore the effects of antioxidants on coumarin polysulfide-induced apoptosis, HCT116 cells were pretreated with 5 mM NAC for 0.5 h prior to exposure to 50 μM SV29.

2.10. Extraction of cellular proteins

Following incubation of HCT116 cells with coumarin polysulfides, cells were resolved in cold phosphate buffered saline (PBS, pH 7.4) and centrifuged together with the cell culture medium at 4 °C and 250×*g* for 7 min. After one washing step with cold PBS, cells were resolved in 50–150 μl RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% sodium desoxycholate, 1% Triton X–100, 0.1% SDS) supplemented with the protease inhibitor cocktail Complete™ according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The cell lysate was left on ice for 15 min, subjected to sonification (3 × 1 min) at 4 °C and then the pellet removed by centrifugation at 16250×*g* at 4 °C for 30 min. The protein content of the supernatant was determined according to the Bradford method using the Bio-Rad protein assay reagent (Bio-Rad, Munich, Germany).

For caspase 3/7 analysis, the modified technique described in Ref. 32 was used. In brief, cells were harvested and extracted for 5 min on ice in lysis buffer (10 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 0.5% NP-40, 10 mM DTT) supplemented with the protease inhibitor cocktail Complete™. Lysates were centrifuged for 10 min at 7,000 rpm at 4 °C and the supernatants were removed and assayed for protein content.

Cytosolic extracts for cytochrome c release were generated by resolving treated cells in cold phosphate buffered saline (PBS, pH 7.4) and centrifuged together with the cell culture medium at 4 °C and 250×*g* for 7 min. The pellets were further resuspended in cold PBS and snap frozen in liquid nitrogen. Cellular fragments were removed by centrifugation at 12500×*g* at 4 °C for 10 min. Extracts were immediately used for Western blot analysis or stored at −20 °C (for the short term) or −80 °C (for the long term).

2.11. SDS–polyacrylamide gel electrophoresis and Western blot analysis

Proteins of total cell extract (50–100 μg) were separated by size using a 12.5% sodium dodecyl sulfate–polyacrylamide gel. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (PVDF) by tank blotting with 20 mM Tris–HCl, pH 8.8, 150 mM glycine as transfer buffer. To ensure equal loading of samples, GAPDH or nucleolin was used as a control. Subsequent immunoblotting was performed by blocking non-specific binding regions of the membrane with 5% skimmed milk in PBS with 0.1% Tween-20 for 1 h at room temperature. The rabbit polyclonal anti-PARP, anti-cdc25C (H6), monoclonal anti-bax, anti-bcl-2, anti-cytochrome *c* and rabbit monoclonal anti-caspase 3 antibodies were each separately used in a dilution of 1:1000 overnight with gentle shaking at 4 °C. The mouse monoclonal p53 (DO-1), rabbit polyclonal GAPDH and the rabbit serum nucleolin antibodies were also used in a dilution of 1:1000 at room temperature for 1 h. After washing (3 × 10 min) the membranes with PBS-Tween-20 containing 1% skimmed milk, they were then incubated with their respective peroxidase-coupled secondary antibodies in appropriate dilutions at room temperature. The membranes were further washed (3 × 10 min) in PBS-Tween-20. Signals were developed and visualized by the Lumilight system of Roche Diagnostic (Mannheim, Germany).

2.12. In vitro phosphatase assay for cdc25

The enzymatic activity of the GST-cdc25 recombinant enzyme was evaluated by fluorimetric assay and was performed in 96-well plates in a specific reaction buffer (50 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, 0.1% BSA, pH 8.1) containing 3-*O*-methylfluorescein phosphate (500 μM) as substrate. The GST-cdc25 proteins, diluted in assay buffer, were used at a final concentration of 1 μg/well. 3-*O*-Methylfluorescein fluorescent emission was measured every 20 minutes for 2 h at 30 °C with a CytoFluor system Perseptive Applied Biosystems; excitation filter: 475 nm and emission filter: 510 nm.

3. Results

Recently we have shown that treatment of tumor cells with diallyl sulfides reduced their viability considerably depending on the length of the sulfur chain.[30–33] Now, we tested the newly designed and synthesized coumarin polysulfides SV25, SV28 and SV29 (Fig. 1A) for their effect on HCT116 cells in different concentrations as 12.5, 25, 50, 100 and 200 M. As a control, we treated cells with the solvent DMSO alone. Cell viability was measured with an MTT assay. As shown in Figure 1B using 25 μM SV25, SV28 or SV29 for 24 h led to reduction in cell viability by 30%, 18% or 22%, respectively. At a concentration of 50 μM, cell viability was reduced to around 50%; at higher concentrations, there was only a slightly further reduction in cell viability. In order to analyze a time dependency of the treatment, cells were incubated with 50 μM SV25, SV28 or SV29 for 8, 24 and 48 h and then cell viability was measured by an MTT assay as shown in Figure 1B and C. The graphs shown in Figure 1C demonstrate that cell viability was already reduced to around 50% after 24 h treatment and further reduced to around 20% viability after 48 h. Thus, these experiments showed a dosage and a time dependent effect of all three coumarin polysulfides.

Furthermore, in our cell viability studies, we found no significant difference between cells treated with SV25, SV28 and SV29; therefore, in subsequent experiments we treated the cells with only SV29 or SV25 and SV29 at different times.

Since reduced cell viability might be due to cell cycle arrest, we addressed the question whether coumarin polysulfide treated cells arrest in G_1- or G_2-phase of the cell cycle. For this, we treated HCT116 cells with 0, 25 or 50 μM SV29 for 24 h. Cells were harvested and incubated with propidium iodide to label DNA. Cells were then analyzed in a cytofluorimeter. As shown in Figure 2, SV29 treated cells clearly accumulated in the G_2-phase (63%). Moreover, cells in sub G_1-phase indicated that cells might also go into apoptosis. Untreated HCT116 cells showed a normal cell cycle profile with about 63% of the cells in G_1-phase, around 5% in S-phase and about 24% of cells in G_2-phase.

Since we detected a sub G_1-fraction, we then analyzed whether coumarin polysulfides might indeed induce apoptosis. Therefore, in this study, we treated HCT16 cells with SV25 and SV29 for different time periods. Cells were then lysed and the cell extract transferred onto a 12% SDS–polyacrylamide gel. After transfer to a PVDF membrane, the filter was incubated with antibodies directed against PARP. Starting at 24 h after treatment of the cells with the coumarin polysulfides, we observed cleavage of PARP with a molecular weight of 116 KDa into the 89 KDa cleavage products (Fig. 3). In agreement with these data, we also observed an increase in the level of γH_2AX, which detects DNA double strand breaks and is a further indication of apoptosis (data not shown). In order to support our conclusion about apoptosis induction, we repeated the experiment described above, but since we found no significant difference between the cells treated with SV25, SV28 and SV29, we

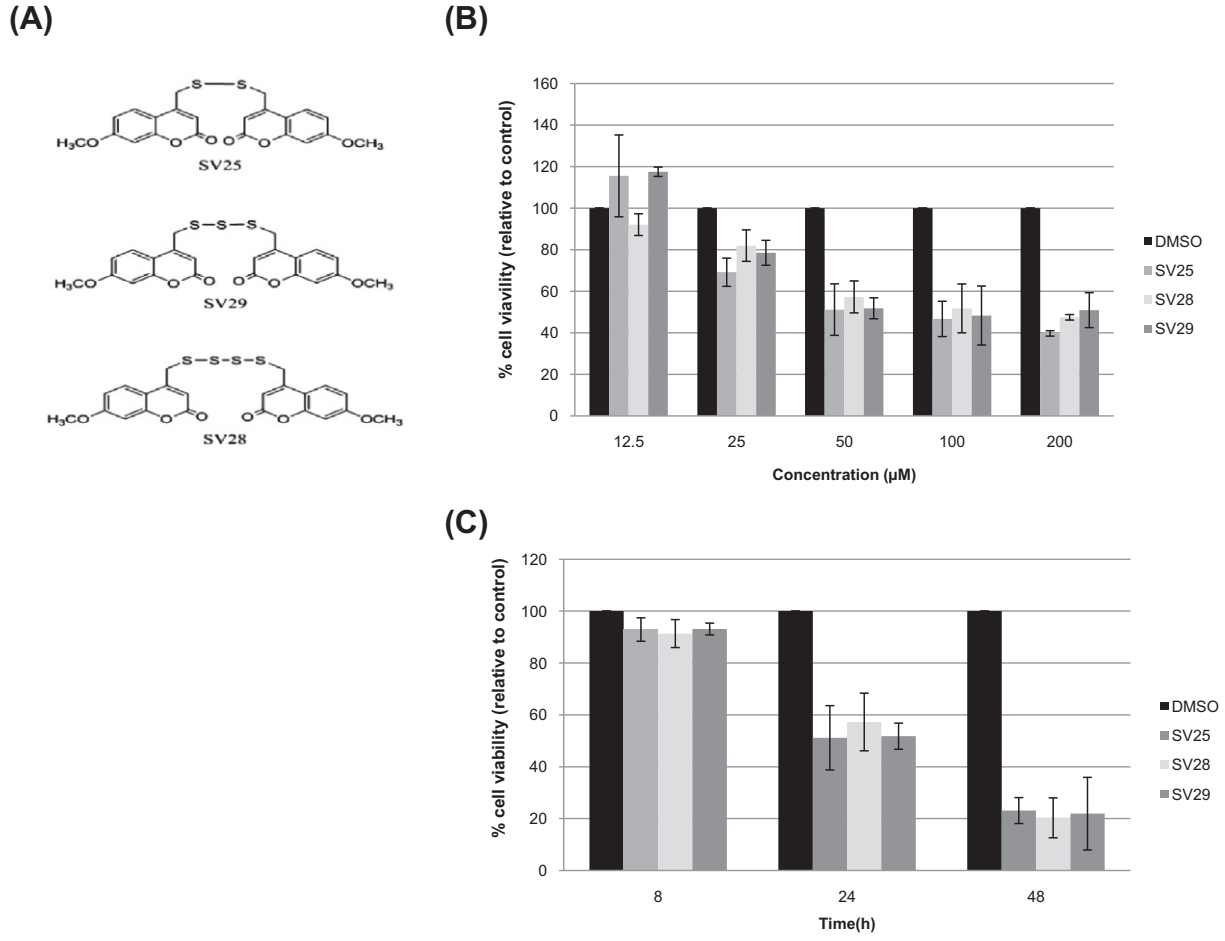
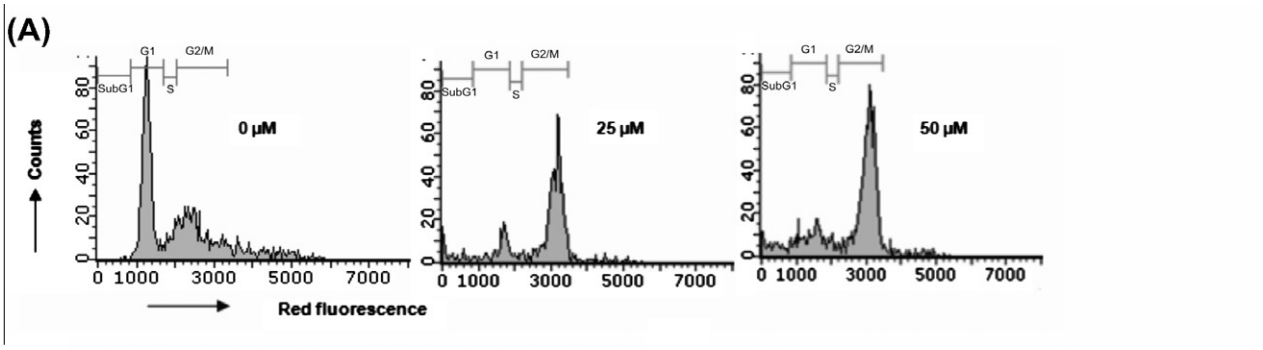


Figure 1. Coumarin polysulfides affect the viability of HCT116 cells in both time and dosage dependent manners. (A) Structures of three of the compounds synthesised—4,4′-disulfanediylbis(methylene)bis(2*H*-chromen-2-one) (SV25), 4,4′-trisulfanediylbis(methylene)bis(2*H*-chromen-2-one) (SV29) and 4,4′-tetrasulfanediylbis(methylene)bis(2*H*-chromen-2-one) (SV28). (B) Cells were seeded in 24-well plates and treated with 0.05% DMSO as a control or 12.5, 25, 50, 100 or 200 μM SV29 for 24 h. Afterwards, MTT assay was done to determine the number of viable cells. (C) Cells were seeded in 24-well plates and treated with 50 μM SV25, SV28, SV29 or 0.05% DMSO for 8, 24 and 48 h. Afterwards, MTT assay was used to determine the number of viable cells as in (B). Data are depicted as means ± SD, (n = 3).



(C)

	G1	S	G2/M	SubG1
0 µM	62.34	4.91	23.45	1.8
25 µM	14.33	4.61	66.07	12.28
50 µM	13.59	3.9	63.19	10.43

Figure 2. Influence of SV29 on cell cycle distribution. HCT116 cells were treated for 24 h with 0, 25 or 50 µM SV29 and then analyzed by FACS. (A) Cytofluorimetric analysis of the treated cells. (B) Percentage distribution of cells in different cell cycle phases. (C) Data for (B). One representative of at least three similar independent experiments is shown here.

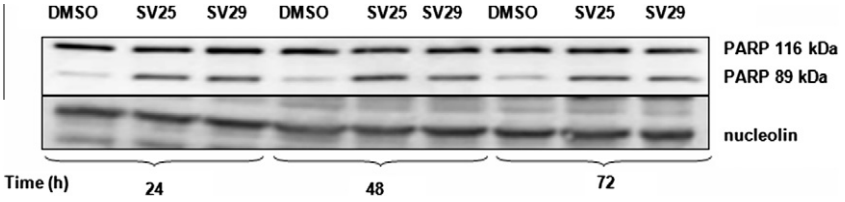


Figure 3. Coumarin polysulfides induce PARP cleavage. HCT116 cells were treated for 24, 48 and 72 h with 0.05% DMSO as a control or 50 µM SV25 or SV29 and cell extracts were treated for PARP cleavage using SDS–polyacrylamide gel electrophoresis and Western blotting. Total protein extract (75 µg) was separated on a 7.5% SDS–polyacrylamide gel, blotted on a PVDF membrane and PARP was detected using the corresponding antibody. Nucleolin was used as a loading control.

performed the experiment with only SV29. Western blot analysis showed a decrease in the level of the anti-apoptotic bcl-2 protein (Fig. 4A), and an increase in the level of the pro-apoptotic bax protein (B) in a time dependent manner. An antibody against GAPDH was used as a loading control. Since mitochondrial cytochrome *c* release is regulated by a decrease in the bcl-2 level, we analyzed the cytochrome *c* release into the cytosol after treatment of the cells with coumarin polysulfides. Accordingly, an increase in cytosolic cytochrome *c* was detected after treatment (Fig. 4C). Finally, we analyzed caspase 3/7 activation after treatment of HCT116 cells with coumarin polysulfides. After 24 and 48 h treatment, we detected an activation of caspase 3/7 (Fig. 4D). LNCaP cells treated with doxorubicin (20 µg/ml) served as a positive control for caspase activation. Thus, from these results we are convinced that apoptosis is induced in HCT116 cells after treatment with coumarin polysulfides. Furthermore, the reduction in bcl-2, the increase in bax, cytochrome *c* release and caspase 3/7 cleavage all clearly argue for the induction of the intrinsic pathway of apoptosis.

Coumarin polysulfides like other polysulfides might induce reactive oxygen species (ROS). In order to evaluate the contribution of ROS to the coumarin polysufide induced apoptosis, we treated HCT116 cells with SV29 in the absence or in the presence of N-acetyl cysteine (NAC). Cells were lysed and then the cell extract analyzed by SDS–polyacrylamide gel electrophoresis followed by Western blot with a PARP specific antibody. As shown in Figure 4E, SV29 induced PARP cleavage already after 24 h and also after 48 and 72 h. In the presence of NAC, however, there was no cleavage of PARP, indicating that NAC prevented apoptosis (Fig. 4E). Thus, these results indicated that ROS might contribute to the coumarin polysulfides induced apoptosis.

In order to evaluate the potential of the coumarin polysulfides, we compared their activities with those of the well characterized diallyl polysulfides (Fig. 5A). We therefore treated HCT116 cells with the coumarin disulfide (SV25) or with the diallyl disulfide (Al_2S_2) or in a second set of experiments with coumarin tetrasulfide (SV28) or with diallyl tetrasulfide (Al_2S_4). After 24 h or 48 h, cell viability was measured with an MTT assay. As shown in Figure 5B, SV25 reduced cell viability more efficiently than the corresponding diallyl disulfide, whereas, the corresponding tetrasulfides exhibited nearly the same activity. Thus, this result indicated that the activity of the coumarin derivates is not dependent on the length of the sulfur chain as it is the case for the diallylpolysulfides. This result also supports the observation shown in Figure 1B.

To analyze whether this behavior might also lead to a more efficient apoptosis induction, we treated HCT116 cells with SV25 or with Al_2S_2. After 24 or 48 h of treatment, cells were extracted and

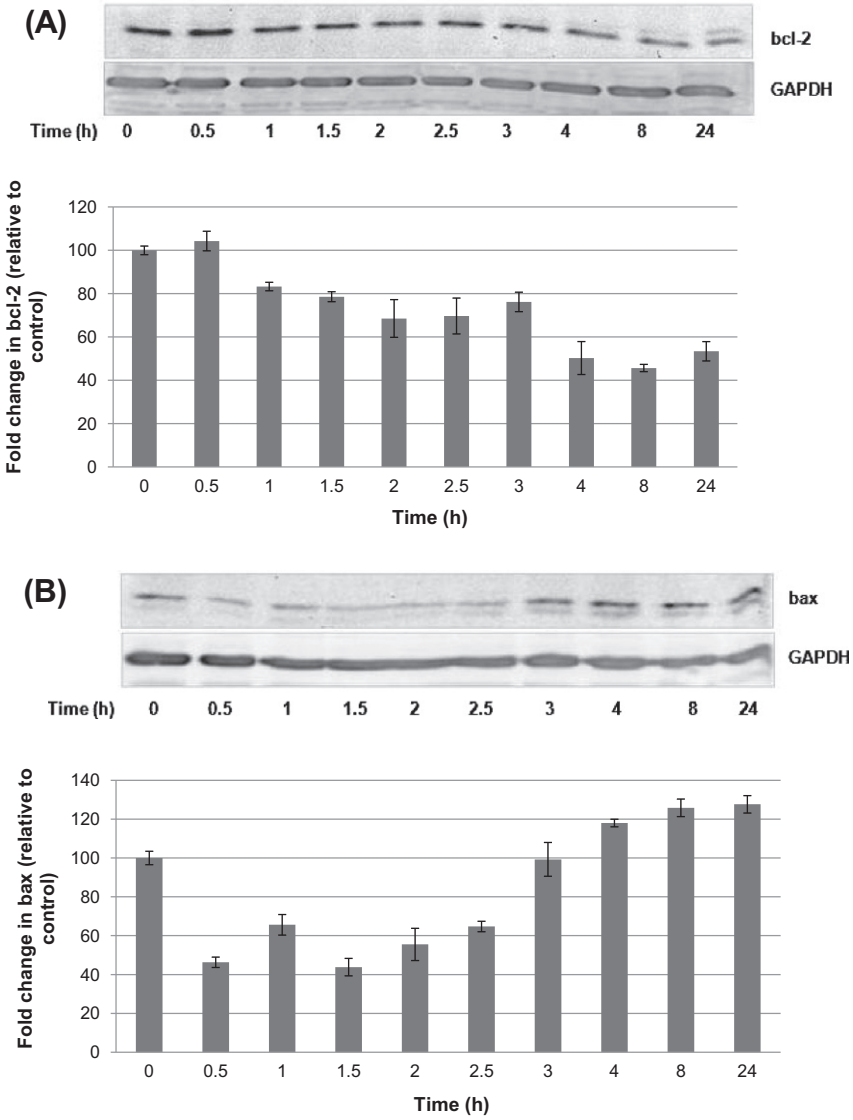


Figure 4. SV29 modulates bcl-2 and bax expression, induces cytochrome *c* release and activates caspase 3/7 in HCT116 cells. HCT116 cells were untreated (0 h) or treated with SV29 for 0.5, 1, 1.5, 2, 2.5, 3, 4, 8 and 24 h, and protein expression was studied by Western blotting. The proteins (50 μg) were separated on a 12.5% SDS–polyacrylamide gel, blotted on a PVDF membrane and bcl-2 (A), bax (B) or cytochrome *c* (C) was visualized with the appropriate antibody. For (D), HCT116 cells were treated with 0 or 50 μM SV29 for 24 and 48 h. As a positive control, LNCaP cells were treated with 20 μg/ml doxorubicin for 24 h or left untreated. Caspase activation was analyzed by Western blot. Cell lysates were separated by electrophoresis as in (A). Full-length caspase 3 and its cleavage products were detected with a caspase 3-specific antibody. One representative of at least 3 Western blots is shown here. NAC pretreatment reduces coumarin polysulfide-mediated apoptosis in HCT116 cells. Pretreatment of HCT116 colon cancer cells with NAC at a concentration of 5 mM for 0.5 h before treatment with 50 μM SV29 prevented PARP cleavage (E). HCT116p53wt cells were treated for 24, 48 and 72 h with 0.05% DMSO, or 50 μM SV29 and cell extracts were treated for PARP cleavage using SDS–polyacrylamide gel electrophoresis and Western blotting. Total protein extract (75 μg) was separated on a 7.5% SDS–polyacrylamide gel, blotted on a PVDF membrane and PARP was detected using the corresponding antibody. Nucleolin or GAPDH were used as a loading control.

the cell extract analyzed on a SDS–polyacrylamide gel followed by Western blot analysis for PARP cleavage. As shown in Figure 5C, the PARP cleavage product was clearly detectable for SV25 after 24 h and even more after 48 h. In contrast only a very faint protein band for the cleavage product was found for Al_2S_2. This result supported the observation that the coumarin disulfide was more active inducing apoptosis than the corresponding diallyl disulfide.

p53 is known to be a key player in the regulation of life and death of a cell. Therefore, we asked whether we could detect an increase in p53 protein expression after incubation of the cells with SV25 and SV29. After incubating the cells for different time periods from 8 to 48 h with SV25 and SV29, cells were lysed and p53 analyzed by SDS–polyacrylamide gel electrophoresis followed by Western blot with a p53 specific antibody (DO1). GAPDH was used as a loading control. Figure 6 shows an increase in the level of p53 over the indicated time periods following treatment.

Since cdc25C is one of the key enzymes responsible for G_2–M phase transition,[34] we tested whether the coumarin polysulfides might influence the level of cdc25C and its activity. HCT116 cells were incubated for various time periods with SV29. Cells were extracted and the cell extract analyzed on a 10% SDS–polyacrylamide gel. Proteins were transferred to a membrane and blotted with the cdc25C specific antibody H6. As shown in Figure 7A, we found a time dependent decrease in the level of cdc25C. In the next step, we analyzed whether coumarin polysulfides might also influence the phosphatase activity. For this type of analysis, we used the human glutathione-S-transferase (GST)-cdc25 recombinant enzyme which was prepared as previously described.[35] We tested the

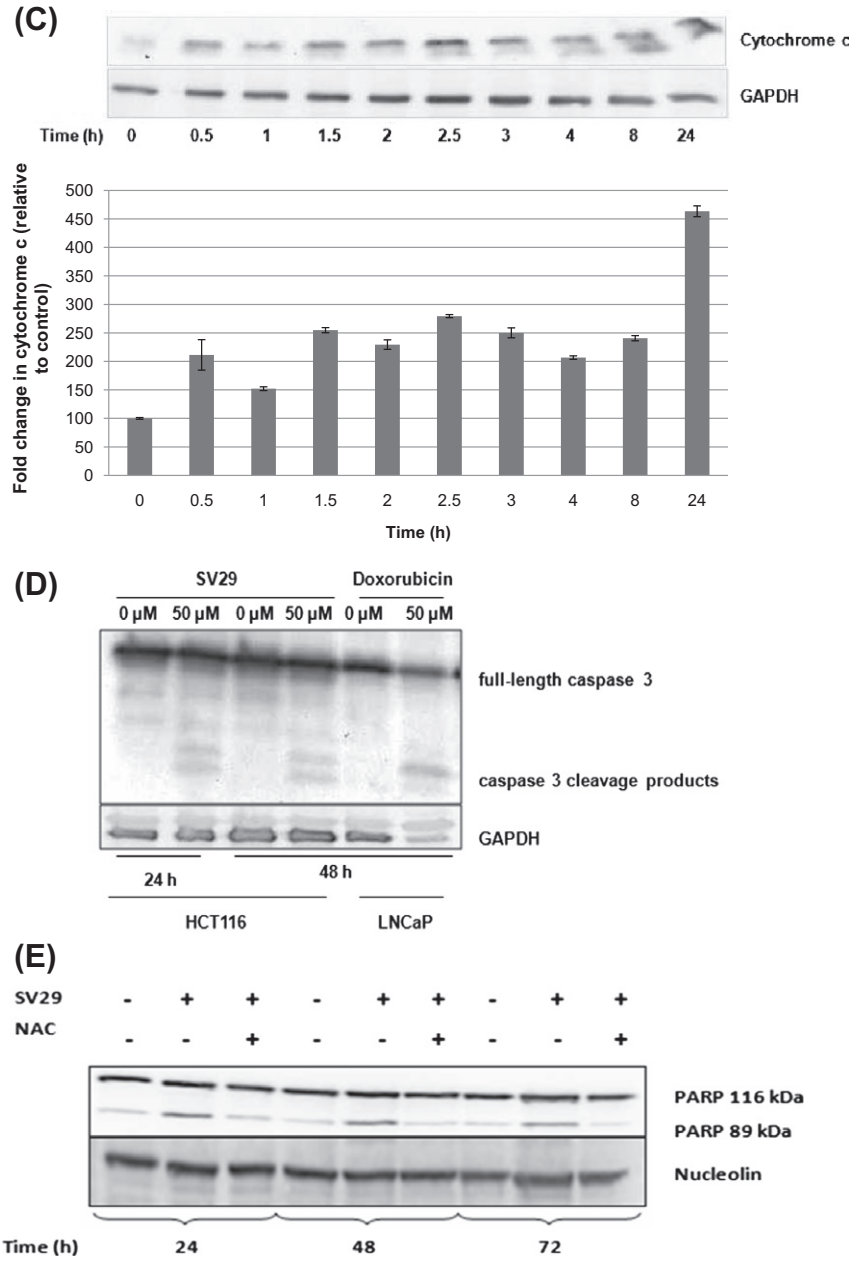


Fig. 4 (*continued*)

inhibitory potential of coumarin polysulfides, SV25, SV28, SV29, and compared this to BN82002 (Sigma–Aldrich), used as reference inhibitor drug. The inhibitory activity was compared to that of DMSO (i.e., expressed as a percentage relative to DMSO control). As shown in Figure 7B, these compounds were able to inhibit cdc25 phosphatase activity. Specifically, the disulfide (SV25) and the trisulfide (SV29) were able to inhibit cdc25A and cdc25C. However, the progressive introduction of sulfur atoms increased the inhibiting activity of such molecules, showing the tetrasulfide to be more potent than the trisulfide, and the trisulfide more potent than the disulfide.

All three polysulfides were less active than the established cdc25 inhibitor BN82002 (Fig. 7B). Nevertheless, we were able to show that the coumarin polysulfides down-regulated the level cdc25C and also the phosphatase activity of cdc25C and cdc25A, which might be both responsible for the G_2-arrest of the cells.

4. Discussion

Coumarins have a long history as anticancer agents (for review see:[17]). On the other hand diallyl polysulfides are among the most studied organosulfur compounds from garlic which are known to be highly effective in affording protection against various cancers in animal models.[26] Most of the experiments published up to now are performed with diallyl sulfide, diallyl disulfide and diallyl trisulfide whereas, the corresponding tetrasulfide was almost recently used. Beside allyl groups, propyl groups were also attached to the sulfur chain.[36] In order to combine the power of coumarins with those of polysulfides, here, for the first time we described the synthesis of dicoumarin polysulfides as di-, tri- and tetrasulfides. Synthesis of the coumarin polysulfides as described here is similar to the method described by Derbesy and Harpp.[37] Treatment of the colon carcinoma cell line HCT116 with coumarin polysulfides,

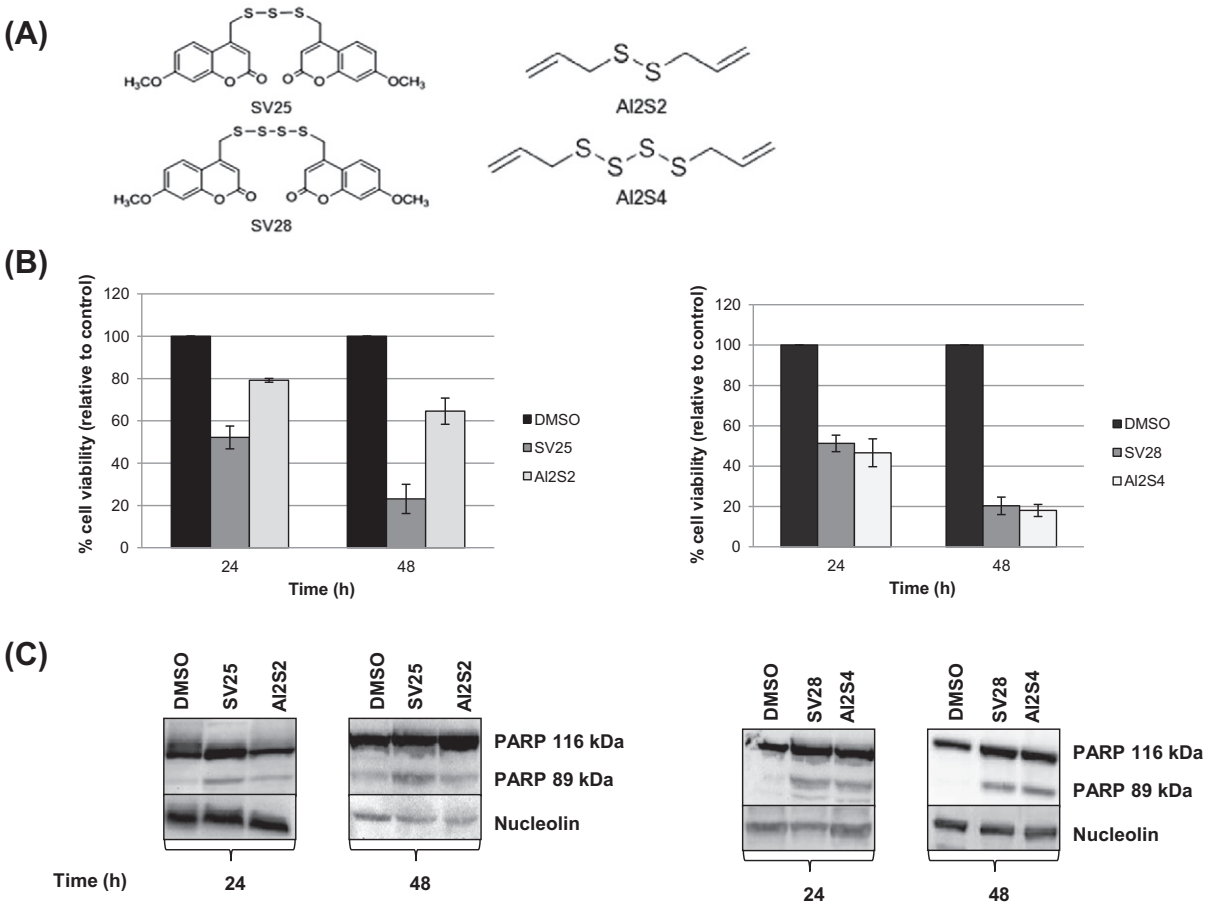


Figure 5. Coumarin polysulfides compared with diallyl polysulfides. (A) structures of two of the newly synthesised compounds—4,4′-disulfanediylbis(methylene)bis(2*H*-chromen-2-one) (SV25), and 4,4′-tetrasulfanediylbis(methylene)bis(2*H*-chromen-2-one) (SV28) with their corresponding well characterized diallyl polysulfides–diallyl disulfide (Al_2S_2) and diallyl tetrasulfide (Al_2S_4). (B), Cells were seeded in 24-well plates and treated with 0.05% DMSO as a control, 50 μM SV25 or 50 μM Al_2S_2 for 24 h or 48 h. In a second set of experiments, cells were treated with 0.05% DMSO as a control, 50 μM SV28 or Al_2S_4 for 24 h or 48 h. Afterwards, MTT assay was done to determine the number of viable cells in both set of experiments as in Figure 1B. Data are depicted as means ± SD, (n = 3). (C), HCT116 cells were treated for 24 or 48 h with 0.05% DMSO as a control or 50 μM SV25 or Al_2S_2. In a second set of experiments, cells were treated with 0.05% DMSO as a control, 50 μM SV28 or Al_2S_4 for the same time periods. (D) Cell extracts were treated for PARP cleavage as in Figure 3. Nucleolin was used as a loading control.

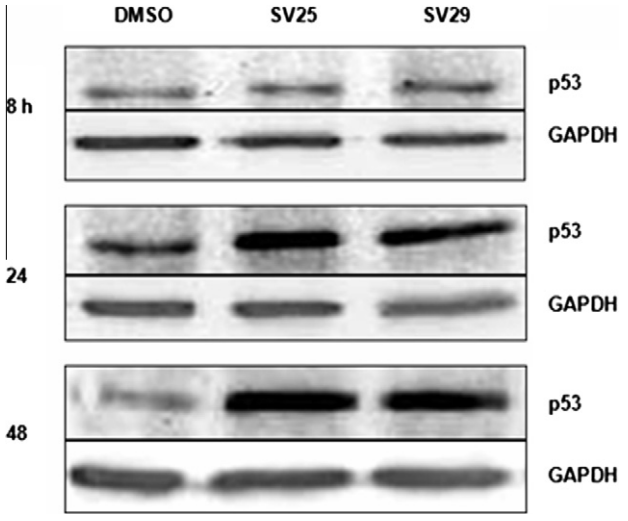


Figure 6. Coumarin polysulfides induce p53 expression in HCT116 cells. HCT116 cells were treated for 8, 24 and 48 h with 0.05% DMSO as a control or 50 μM SV25 or SV29, and p53 protein expression was studied by Western blotting. Total proteins (50 μg) from the cell extract were separated on a 12.5% SDS–polyacrylamide gel, blotted on a PVDF membrane and p53 was visualized with the mouse monoclonal p53 (DO-1) antibody. One representative of at least 3 Western blots is shown here.

resulted in reduced cell viability as shown in Figure 1. The coumarin disulfide, trisulfide and the tetrasulfide were nearly equally active, whereas, in the case of the diallyl polysulfides, the trisulfide is more active than the disulfide and the tetrasulfide is more active than the trisulfide. These differences in the activities were shown in a direct comparison of the corresponding disulfides and tetrasulfides. The coumarin disulfide was considerably more active than the corresponding diallyl disulfide, whereas, the activities of the coumarin tetrasulfide and that of the diallyl tetrasulfide are comparable. All three coumarin derivatives acted in a time and concentration dependent manner.

A decrease in cell viability may result in apoptosis which may be induced via an extrinsic or an intrinsic or mitochondria-mediated pathway. The intrinsic pathway is regulated by the anti-apoptotic bcl-2 family of proteins and by the pro-apoptotic bax protein. Here, we show a decrease of the level of bcl-2 and an increase in the level of bax after incubation of HCT116 cells with coumarin polysulfides indicating that the coumarin polysulfides act via the intrinsic pathway of apoptosis. Moreover, we observed a cytochrome *c* release from the mitochondria after treatment of HCT116 cells with coumarin polysulfides, further supporting the idea of the induction of the intrinsic pathway of apoptosis. Apoptosis was further documented by PARP cleavage as a final step in apoptosis. These observations are in agreement with the results

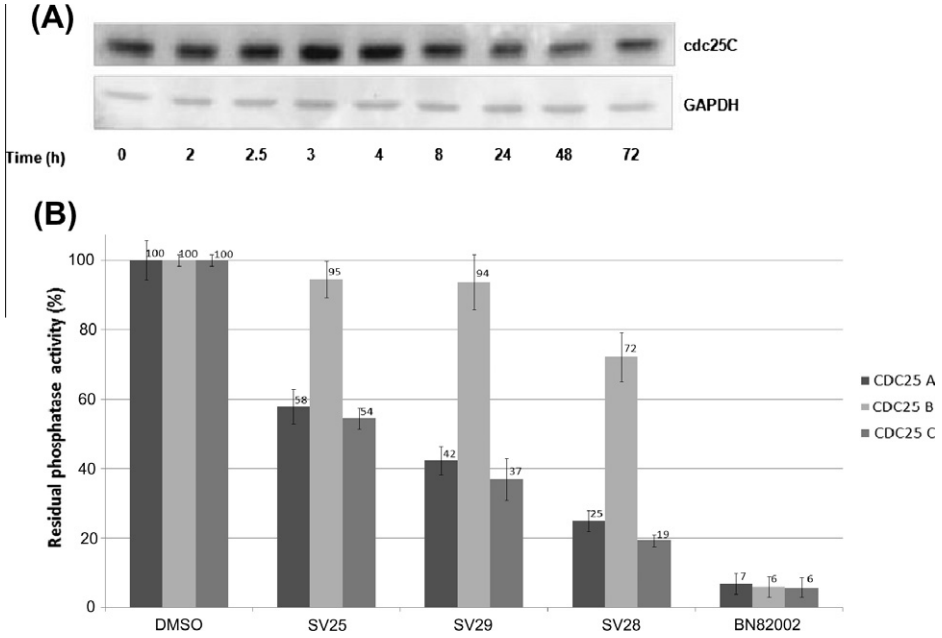


Figure 7. Coumarin polysulfides down-regulate cdc25C expression in HCT116 cells and inhibit the phosphatase activity of recombinant cdc25C phosphatase. (A) HCT116 cells were treated with 50 μM SV29 and cdc25C protein expression was determined by Western blotting. Total protein extract (50 μg) was separated on a 12.5% SDS–polyacrylamide gel, blotted on a PVDF membrane and cdc25C was detected using anti-cdc25C (H6) antibody. GAPDH was used as a loading control. (B) Residual activity of cdc25 phosphatases was measured by a dephosphorylation assay with 3-*O*-methyl fluorescein phosphate following incubation with 100 μM SV25, SV29 and SV28. DMSO treated cells were set 100%.

showing that bcl-2 overexpression confers protection against diallyl polysulfide induced apoptosis[38] at least in prostate cancer cells.

The apoptotic effects of the polysulfides seem to be in part due to their capacity to cause oxidative damage by increasing the production of reactive oxygen species. Diallyl disulfides can undergo a network of different redox reactions as described by Jacob.[39] These redox reactions can include one- or two-electron and radical reactions. Furthermore, polysulfides can coordinate metal ions. Here we show that *N*-acetyl-cysteine-(NAC) prevented the coumarin polysulfide induced apoptosis.

There are contradictory results concerning the role of p53 in diallyl polysulfide induced apoptosis in human colon cancer cells. Song et al. reported no change in the expression level of p53 by less than 12 h post-exposure[40] whereas Busch et al. found an increase in the p53 level.[30] Furthermore, down-regulation of p53 siRNA prevented apoptosis after diallyl polysulfide treatment. Here, we have shown that coumarin polysulfides induced an increase in the p53 level between 8 and 72 h after treatment. On the other hand, HCT116 cells lacking p53 were also stimulated to apoptosis by diallyl polysulfides indicating that p53 might be implicated in apoptosis induction but it also seems to be dispensable.[30]

Knowles and Milner were the first to show that diallyl disulfide caused an accumulation in the G_2/M phase of the cell cycle.[41,42] Here, we show that the coumarin polysulfides also caused a growth arrest in the G_2-phase of HCT116 cells. We further show that the growth arrest was accompanied by a decrease in the level of the cdc25C phosphatase which is in agreement with data published for diallyl disulfide.[30,42,43] In addition to the down-regulation of the level of cdc25C, we also found that coumarin polysulfides directly inhibited the phosphatase activity of purified cdc25C and also the other family members, namely cdc25A and cdc25B. As shown by Viry et al.[44], this inhibitory activity increased with the length of the sulfur chain. cdc25C phosphatases contain an active-site cysteine. It has been shown that redox reactions of active-site cysteines serve as a form of reversible regulation of cdc25s. Furthermore, it has been shown that the active-site cysteines of cdc25s are highly susceptible to oxidation.[45] Thus, it might well be that coumarin polysulfides directly target the active centres of the cdc25 family of phosphatases. Down-regulation of the cdc25s as well as inhibition of the phosphatase activities are probably both responsible for the observed growth arrest of the cells in G_2-phase of the cell cycle.

5. Conclusion

The newly synthesized di-coumarin polysulfides are potent agents which induce cell cycle arrest in the G_2/M phase. This cell cycle arrest is due to a down-regulation of the level and the activity of cdc25C. Furthermore treatment of colon cancer cells with these new polysulfides resulted in the induction of the intrinsic pathway of apoptosis. Thus, coumarin polysulfides are promising new anticancer agents.

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